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Synthesis and biological evaluation of 21-arylidenepregnenolone derivatives as neuroprotective agents

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ABSTRACT

A series of 21-arylidenepregnenolone derivatives and their corresponding epoxides were synthesized. The neuroprotective effects of these steroidal compounds against amyloid-β_{25-35} ($A\beta_{25-35}$)- and hydrogen peroxide (H_2O_2)-induced neurotoxicity in PC12 cells, and oxygen–glucose deprivation (OGD)-induced neurotoxicity in SH-SY5Y cells were evaluated. The bioassay results indicated that several 3β-pregn-21-benzylidene-20-one derivatives displayed potent in vitro neuroprotective effects in different screening models, for example, compounds **2b**, **3a**, **3b**, and **3s** showing significant activities against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells, **2b** showing significant activities against H_2O_2-induced neurotoxicity in PC12 cells, and **2g**, **3b**, and **3e** showing potent protection against OGD insult. The results suggested that introduction of an arylidene group into steroidal nucleus played an important role in neuroprotective activity, while the formation of epoxy group at C-5,6 could be also important for the neuroprotective activity in some degree. The pharmacological data reported here are helpful for the design of novel steroidal neuroprotective candidates.

Neurologic diseases, such as Alzheimer's disease (AD),[1] are a class of disorders with complex contributing factors. Although the histopathogenesis of AD is still unknown, it is hypothesized that the accumulated amyloid-β-peptide (Aβ), triggering critical intracellular signaling pathways that lead to cell stress and apoptosis, is considered as one of the original cause of AD.[2] Moreover, cerebral ischemia is characterized by insufficient glucose and oxygen supply which result in imbalanced energy metabolism and at last cell death, which is also a cause of AD.[3,4] In addition, oxidative stress is commonly observed and may contribute to the progress of AD,[5] which may result from excessive reactive oxygen species (ROS) production and insufficient antioxidant defense systems. H_2O_2 is the main form of ROS, which causes protein and lipid peroxidation and DNA damage and at last cell death.[6] To date, although acetylcholinesterase inhibitors (e.g., donepezil, galantamine) exert beneficial role in improving AD symptom, no effective treatment has been proved to stop the progressing of AD and cerebral ischemia. Therefore, discovering novel compounds with multi-effects may be an effective strategy for the treatment of the AD disease.

Steroids are a large group of secondary metabolites widely presented in animals and plants. Many steroids were reported to display various bioactivities and great application potentials. In particular, some of them have been used in both clinical and preclinical studies.[7,8] Well known steroids pregnenolone (**1**) and dehydroepiandrosterone (DHEA), which are synthesized in the central nervous system, were discovered to have neuroprotective properties. It was proposed that the aromatization of **1** and DHEA mediated by aromatase to estradiol, which also has neuroprotective effect, might be part of an endogenous mechanism for neuroprotection.[9] In addition, a literature survey revealed that some synthetic or naturally occurring steroids (selective examples of steroids are shown in Fig. 1), such as 17β-alkoxyestra-1,3,5(10)-trienes,[10a,b] spiro-epoxyneurosteroid derivatives,[10c] 22*R*-hydroxycholesterol (SP222) and SP233,[10d,e] had been demonstrated to have promising neuroprotective activity. Moreover, methyl (20*R*)-3β-hydroxy-20-(3-methyl-2-butenyl)-pregnan-21-oate,[11] which is a synthetic analog of methyl spongoate isolated from Sanya soft coral *Spongodes* sp.,[12] was also found to show significant neuroprotective activity against H_2O_2-induced neurotoxicity in PC12 cells. All these analogs mentioned above possess the same conventional steroidal skeleton, differing from each other only at the side chain attached to C-17 of steroidal nucleus. Meanwhile, it was reported that introduction of aromatic group could play an important role in the activity of steroids.[13,14] Based on the above observation and with the purpose of searching for new steroidal neuroprotective agents, a series of pregnenolone derivatives with different arylidene groups at C-21 and epoxy group at C-5,6 was prepared, and evaluated for their biological activities.

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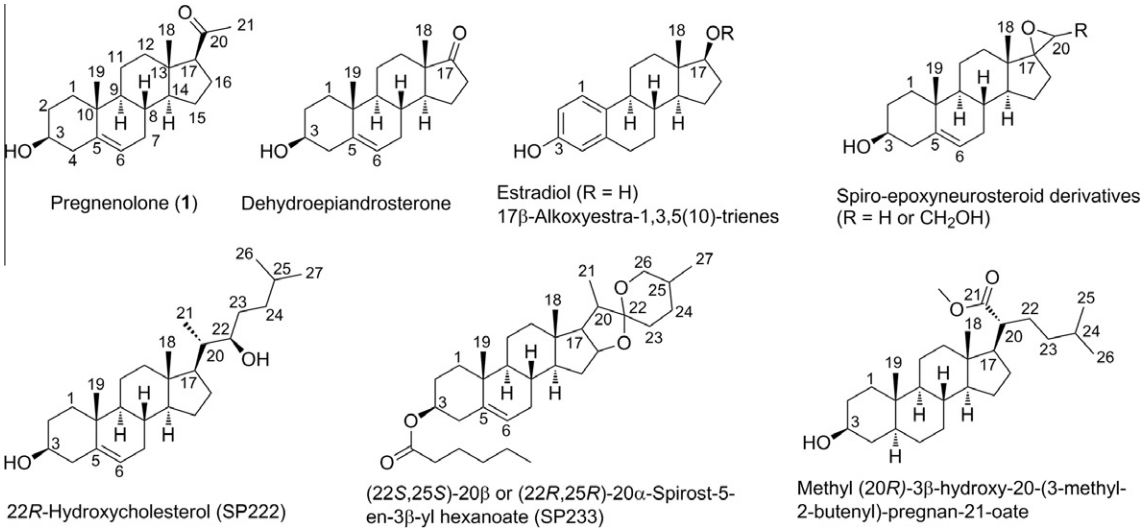
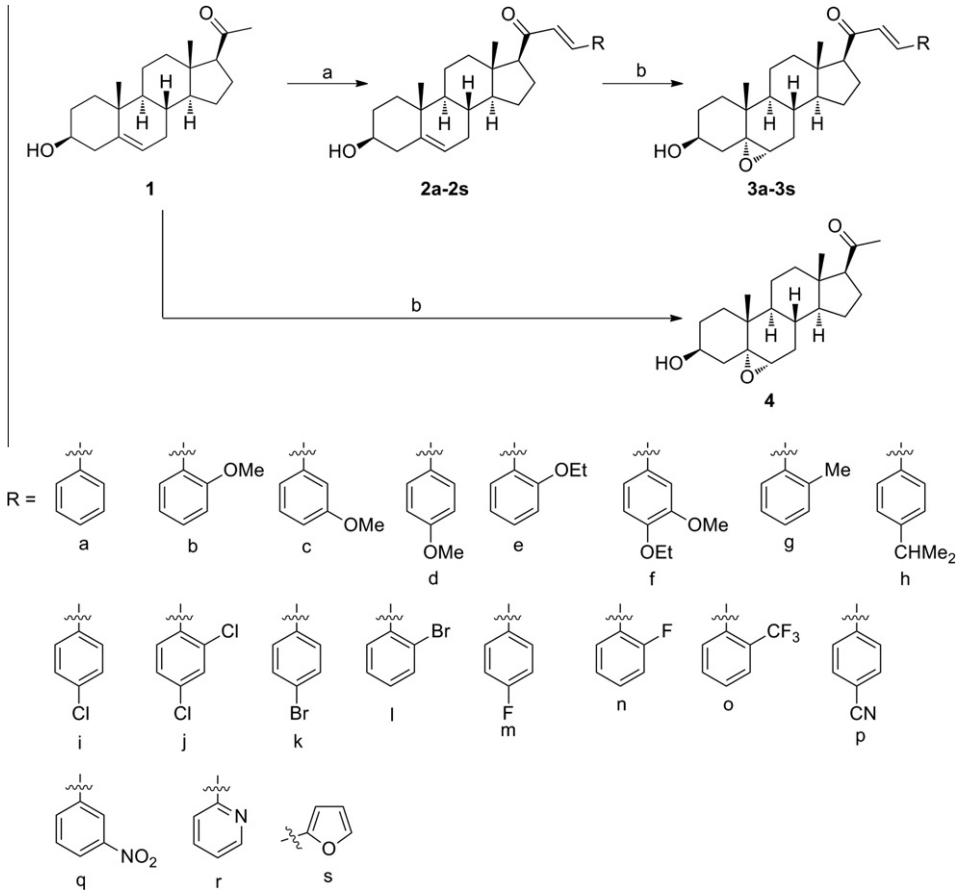


Figure 1. Compounds reported with neuroprotective activity.

The synthetic route was outlined in Scheme 1. In a Claisen–Schimidt condensation reaction, compound **1** was reacted with aromatic aldehydes in 95% EtOH for 24–48 h to afford α,β-unsaturated ketones **2**.[14,15] In **2**, the coupling constants of the H-21 with H-22 ($^2J_{21\text{-H},22\text{-H}} \approx 16$ Hz) indicates that the double bond had (*E*) geometry. Oxidation of the δ-5,6 double bond of **2a–3s** and **1** with mCPBA in CH_2Cl_2 at 0 °C for 0.5 h afforded epoxides **3a–s** and **4**, respectively.[16] In the ^{1}H NMR spectra of derivatives **3** and **4**, the splitting pattern and the coupling constants of H-6 with H_α-7 (d, $J_{6\text{-H},7\text{-H}\alpha} \approx 4.2$ Hz), similar to that of reported 5α,6α-epoxysteroids,[17] indicated that the 6-H has β orientation and hence the epoxide oxygen is α-oriented.

In order to discover potential neuroprotective leads, all compounds were evaluated for their in vitro neuroprotective effects against $A\beta_{25-35}$ (the toxic fragment of Aβ)-induced damage in PC12 cells.[18] The results are showed in Table 1. Compared with



Scheme 1. Synthesis of derivatives **2**–**4**. Reagents and conditions: (a) solid KOH, 95% ethanol, rt, 24–48 h, 85–95%; (b) mCPBA, CH_2Cl_2, 0 °C, 0.5 h, 80–90%.

Table 1
Neuroprotective effects of all compounds against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells

Compd	Cell viability[a] (%)		Compd	Cell viability (%)	
	10 μM	1 μM		10 μM	1 μM
2a	29.0	N.A.[b]	**2k**	N.S.	N.S.
3a	70.5	64.8	**3k**	32.5	50.1
2b	74.5	64.1	**2l**	N.A.	N.A.
3b	73.7	N.A.	**3l**	61.2	N.A.
2c	44.0	50.3	**2m**	32.6	N.A.
3c	N.A.	N.A.	**3m**	47.6	N.A.
2d	25.7	N.A.	**2n**	38.3	53.1
3d	29.6	N.A.	**3n**	36.7	N.A.
2e	N.A.	N.A.	**2o**	N.A.	N.A.
3e	49.5	N.A.	**3o**	N.A.	N.A.
2f	26.7	N.A.	**2p**	N.S.	N.S.
3f	N.A.	61.1	**3p**	N.A.	N.A.
2g	50.4	N.A.	**2q**	37.8	51.2
3g	49.9	N.A.	**3q**	31.3	N.A.
2h	34.9	N.A.	**2r**	N.A.	N.A.
3h	49.8	N.A.	**3r**	29.7	N.A.
2i	N.S.[c]	N.S.	**2s**	36.9	52.5
3i	39.1	N.A.	**3s**	82.7	N.A.
2j	34.5	N.A.	**4**	N.A	N.A.
3j	6.0	N.A.	**1**	N.A	N.A.
			EGCG	65.4	N.T.[d]

[a] The neuroprotective effects of these compounds on $A\beta_{25-35}$-induced neurotoxicity in PC12 cells. The cell viability in control was taken as 100%, and the average value of cell viability under $A\beta_{25-35}$ exposure was 54.3% ± 3.61. The value of cell viability lower than 57.6% is considered to be cytotoxic. The positive control is epigallocatechin gallate (EGCG).
[b] N.A. means not active.
[c] N.S. means not soluble.
[d] N.T. means not tested.

inactive **1** and **4**, most derivatives of **2** and **3** displayed neuroprotective activity at 10 μM, indicating that the introduction of arylidene group into steroidal nucleus had an important effect on bioactivity. Though many of derivatives **2** and **3** showed interesting neuroprotective effects, unfortunately, some of them possess unexpected cytotoxicity toward PC12 cells. Indeed, many natural or synthetic compounds with α,β-unsaturated carbonyl group (Michael acceptor) had been also reported to have cytotoxicity,[12,19] and Michael acceptor moieties probably can form adducts with reactive thiol groups of proteins causing change of protein conformation, which might be responsible for the cytotoxicity against cell lines,[20] but it was unclear that whether the cytotoxicity of derivatives **2** and **3** originates from α,β-unsaturated carbonyl functionality. Among the derivatives **2**, only *ortho*-methoxyl substituted compound **2b** exhibited promising neuroprotective effect, even at 1 μM (cell viability: 74.5 at 10 μM and 64.1 at 1 μM, respectively). Compared with **2b**, the alkoxyl group substituted derivatives, **2c**, **2d**, and **2f**, showed no potent neuroprotective effect, indicating that the *ortho*-substituent might be important for neuroprotective effect against $A\beta_{25-35}$ in PC12 cells. The *ortho*-ethyl substituted compound **2e** and other derivatives **2** with *ortho*-halogen, trifluoromethyl substituents also showed no neuroprotective effect. This result not only indicated that the hindrance effect of substituent could impact the neuroprotective effect, but also the neuroprotective effect appears to be increased when there is an electron-releasing substituent on the benzene ring. Among the derivatives **3**, *ortho*-methoxyl substituted compound **3b** also exhibited promising neuroprotective effect (cell viability: 73.7 at 10 μM), almost showing the same high activity as **2b**. It worth to note that compound **3s**, bearing a furan ring with smaller hindrance effect, had significant activity (cell viability: 82.7% at 10 μM). Another compound **3a** with a non-substituted benzene ring also exhibited potent neuroprotective effect (cell viability: 70.5% at 10 μM, and 64.8% at 1 μM, respectively). Compared with

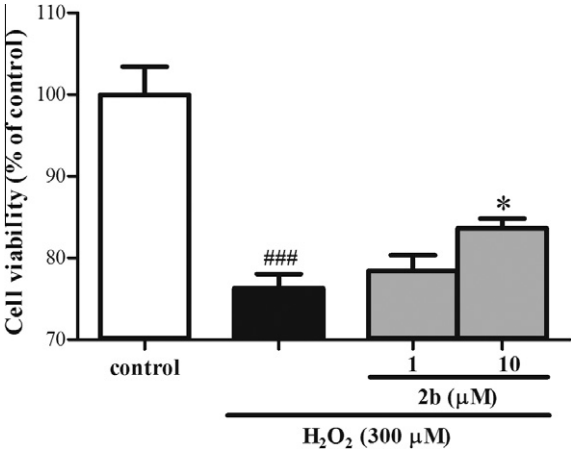


Figure 2. Neuroprotective effect of **2b** on H_2O_2-induced injury in PC12 cells. **2b** at 10 μM significantly attenuated the reduced cell viability. Data are expressed as mean ± SD (n = 3–6), $^{###}P$ <0.001 versus control, $^{*}P$ <0.05 versus H_2O_2 treated only.

that of **2**, the bioactivity of most derivatives of **3** did not dramatically change, but there seems to be ambiguous relationships between the introduction of the epoxide group and the increased neuroprotective effect of **3a**, **3l**, and **3s**, when compared with that of their precursors.

All compounds were further subjected to bioassay to test their in vitro neuroprotective effects against H_2O_2-induced damage in PC12 cells, and against OGD-induced neurotoxicity in SH-SY5Y cells (as an in vitro stoke or AD model).[18] The results are shown in Figures 2 and 3, respectively. Among the tested compounds, only compound **2b** exhibited significantly neuroprotective effect at 10 μM (cell viability: 83.7%) on H_2O_2-induced damage in PC12 cells. Compound **3b** showed potent protection against OGD insult with cell viability of 98.7% at 10 μM, while **3e** showed potent protection against OGD insult at 5 μM (cell viability: 98.8%) and **2g** had a little decreased activity (cell viability: 84.8% at 5 μM). It is interesting to note that all these four active compounds were substituted with electron-releasing groups at the *ortho*-position of benzene ring. Compounds with substituents at *meta* or *para* positions or electron-withdrawing groups at ortho-position were inactive. This indicated that the *ortho*-substituent with electron-releasing effect in benzene ring, such as *ortho*-alkoxyl or methyl

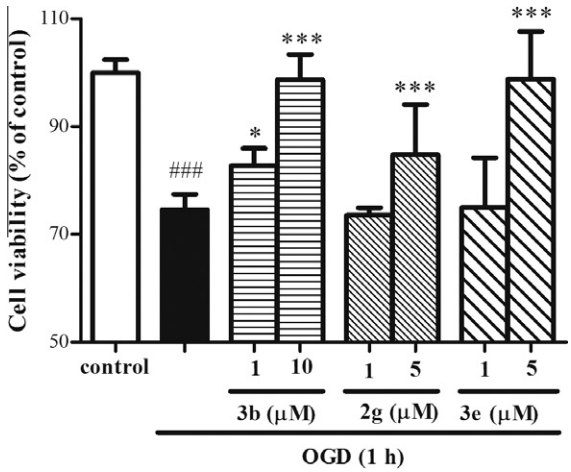


Figure 3. Neuroprotective effects of **3b**, **2g**, and **3e** on OGD-induced injury in SH-SY5Y cells. Compounds **3b**, **2g** and **3e** show potent protection against OGD insult. Data are expressed as mean ± SD (n = 3–6), $^{###}P$ <0.001 versus control, $^{*}P$ <0.05, $^{***}P$ <0.001 versus OGD treated only.

group might be required for the neuroprotective effects against OGD-induced neurotoxicity in SH-SY5Y cells.

In summary, a series of 21-arylidenepregnenolone derivatives was synthesized and biologically evaluated. The bioassay results indicated that several synthetic derivatives displayed potent neuroprotective effects in different screening models, e.g., compounds **2b**, **3a**, **3b**, and **3s** showing significant activities against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells, **2b** showing significant activities against H_2O_2-induced neurotoxicity in PC12 cells, while **2g**, **3b**, and **3e** showing potent protection against OGD insult. The results observed in present studies indicated that introduction of an arylidene group into steroidal nucleus probably play an essential role in neuroprotective activity, and the formation of epoxy group at C-5,6 could be also important for the neuroprotective activity in some degree. Our preliminary structure–activity relationship (SAR) study provided information that could be useful for the design of novel steroidal neuroprotective drug candidates or leads. Further studies to improve neuroprotective activity and clarify the neuroprotective mechanism of this class of compounds are in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.103.

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18. *Materials and Methods:* SH-SY5Y and PC12 cells were high passages from the American Type Culture Collection and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. PC12 cells were pretreated with compounds for 2 h and then suffered cell injury by 1 μM $A\beta_{25-35}$ or 300 μM H_2O_2 for another 24 h; SH-SY5Y cells, pretreated with compounds for 2 h, were exposed to 1 mg/mL OGD for 1 h and cultured for another 24 h under normal condition and SH-SY5Y cells cultured with glucose under normal condition were served as control. Cell viability was evaluated by incubating with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 3 h under 5% CO_2/95% air at 37 °C. Media were replaced with 100 μl DMSO, then absorbance was read at 490 nm. Data were analyzed by one-way analysis of variance (ANOVA) and expressed as means ± SD with P <0.05 as significance.
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